



Ethyl Acetate Fraction of *Caesalpinia sappan* L. Enhances Cisplatin's Cytotoxicity on HeLa Cells via G1 and S Arrest through p53 Expression

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Abstract

Cisplatin (cisp) is the first line chemotherapeutic agent for several cancer diseases which can cause significant side effects and cellular resistance. Combination-chemotherapy treatment (co-chemotherapy) was reported to be able to reduce cisp effects. Therefore, this study was carried out to investigate the cytotoxic activity of ethyl acetate fraction of *C. sappan* (EFC) in combination with cisp by observing apoptosis induction and cell cycle profile. Cytotoxic activity was evaluated by MTT assay. Cell cycle and apoptosis analysis were performed using flow cytometry and p53 expression was analyzed using immunocytochemistry. EFC performed cytotoxic effect on HeLa cells by showing morphological changes such as cell shrinkage, rounding and decreasing of cells viability in concentration dependent manner, giving IC₅₀ value of 65 µg/mL. Combination of EFC and cisp in low concentration decreased cell viability into 36.86%. Further assay indicated that this combination caused redistribution of cell cycle arrest in G1 and S phases through p53 stabilization in nucleus. However, that mechanism was not followed by apoptosis. These results provide evidence to support EFC development as the enhancer of cisp effect, by improving its cytotoxicity on HeLa cells. EFC increases HeLa cells sensitivity to cisp through G1 and S cells' arrest depending on p53 expression.

Keywords: co-chemotherapy, EFC, cervix cancer HeLa cells, p53, G1 and S arrest.

INTRODUCTION

Cervical cancer is the third most common disease among women worldwide (Jemal, *et al.*, 2011), which 70% of its occurrence is caused by HPV infection (Castellsagué, 2008). Globally, 80-85% of death occurs in low- and middle-economic-income countries. The statistics estimation was made based on Globocan 2008 which state that the incidence of cervical cancer is increasing every year, with global estimation of new cases expected to increase 2% per annum to 770,000 new cases by 2030 (Forman, *et al.*, 2012). There are several ways to treat cervical cancer, including surgery, radiation therapy, chemotherapy, and combination of those treatments. To date, chemotherapeutic agent is the most common treatment (Martin, *et al.*, 2013; Leaver and Labonte, 2010).

Cisplatin (cisp) is the first chemotherapeutic agent from platinum (Jamieson and Lippard, 1999). This agent caused many side effects, such as severe nausea and vomiting, acute and chronic nephrotoxicity, anemia (Miller, *et al.*, 2010; Rabik and Dolan, 2007), acute cochlear toxicity (ototoxicity) (Mukherjea and Rybak, 2011) and also developed cells resistance (Sirichanchuen, *et al.*, 2012). Some of natural compounds were known as cytotoxic agent and can be combined with chemotherapeutic agent. Therefore, cisp's side effects and cellular resistance caused by cisplatin can be reduced using chemotherapeutic combination with natural agent by decreasing of cisplatin uses (Zhang, *et al.*, 2011; Zhao, *et al.*, 2004).

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Herbal medicines used in nutrient supplements as anti-cancer and anti-inflammatory agents give benefit for patients (Ho, *et al.*, 2002) to overcome side effects and drug resistance (Zhang, *et al.*, 2011). The heartwood of *Caesalpinia sappan* has been used traditionally for medical treatment in Asia, consumed as a drink or a dye (Badami, *et al.*, 2004). Previous studies showed that *C. sappan* extract (Ueda, *et al.*, 2002) and its isolated compounds, brazilin and brazilein, potentially exhibited antitumor activity on several rodents and tumor cancer cells (Ren, *et al.*, 2011; Yen, *et al.*, 2011), but not on normal epithelial cells (Lee, *et al.*, 2010). The main constituent of *C. sappan*, brazilin, can be oxidized by air and light to produce brazilein that is soluble in ethyl acetate (Kim, *et al.*, 1997). Therefore, in this study, the effects of EFC-single treatment and in combination with cisp on HeLa cells were determined. Observation was done by observing cell viability, apoptosis induction, and cell cycle progression. Expression of p53 protein level was also investigated. More research in field of science will help for further explore the effectiveness and safety approaches, as both agents in conventional treatment and herbal medicine.

METHODS

Preparation of EFC

Caesalpinia sappan heartwood powder was obtained from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT), Tawangmangu, Indonesia. *C. Sappan* wood was extracted with methanol, evaporated, and partitioned with *n*-hexane and ethyl acetate by liquid-liquid extraction. Ethyl acetate fraction was evaporated by using rotary evaporator to get the residue as EFC. The dried fraction was re-solubilized in dimethyl sulfoxide (DMSO) before used as treatment sample. Cisplatin (Wako, $\geq 98\%$ HPLC, Japan, CAS No.033-20091) was used as chemotherapeutic agent.

Chemicals and reagents

DMEM (Dulbecco's Modified Eagle Media, low glucose), FBS (Fetal Bovine Serum), penicillin/streptomycin, fungizone were purchased from Gibco (Invitrogen, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide), PI (Propidium Iodide) (minimum 95% (HPLC) were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). DMSO (99.5% pro GC, Sigma Aldrich Chemie GmbH, Steinheim, Germany), trypsin-EDTA (Gibco, Invitrogen, Canada), FITC-Annexin V Apoptosis Detection Kit (Biovision, USA), Triton-X (triton X-100 for GC, E.Merck, 64271, Darmstadt, Germany). SDS (Sodium Dodecyl Sulphate), HCl were purchased from Merck, Germany. RNase was obtained from Lab. Animal Sciences, NAIST, Japan. All other chemicals were used under analytical grade.

Cell culture

HeLa cells were obtained from Prof. Masashi Kawaichi, Laboratorium of Gene Function in Animal, Graduate School of Biological Science, Nara Institute of Science and Technology (NAIST), Japan. Cells were maintained in DMEM-low glucose, supplemented with 10% (v/v) FBS, 1.5% (v/v) penicillin-streptomycin, and 0.5 % (v/v) fungizone. Cells were incubated at 37°C and 5% CO₂.

MTT assay

HeLa Cells were grown 12×10^3 onto 96-well plates before being exposed to drug treatment. For cell viability assay, cells were treated for 24 h with increasing concentration of EFC-single treatment (0, 30, 40, 50, 60, 70, 80, 90 $\mu\text{g/mL}$). Combination treatment was done using both of EFC and cisp, incubated for 24 h. As negative control, the cells were only treated with growth medium. At 100 μL media containing MTT (0.5 mg/mL) was added to each well then incubated for 3 h at 37°C. The reaction was stopped by dilution with 10% (w/v) SDS in 0.01 M HCl, then continued by incubation for overnight. The absorbance was determined by using ELISA reader (Bio-Rad microplate reader Benchmark, serial no. 11565, Japan) at λ 595 nm as previously described with some modification (Mosmann, 1983).

Flow cytometry

FACS analysis was carried out to investigate apoptosis quantification and cell cycle distribution. Around 5×10^5 cells/well were grown in 6-well plates and cells were treated with EFC, cisp, and combination of both compounds for 24 h.

Trypsinized adherent cells were collected and were prepared for detection. Cell apoptosis was detected by using a FITC-Annexin V Apoptosis Detection Kit (Biovision, USA). Then, apoptosis was analyzed by flow cytometry (BD FACS-Calibur, USA).

For cell cycle analysis, the trypsinized adherent cells were collected and detected by adding flow cytometry reagents (25µL PI, 2.5µL RNase, 0.5µl Triton-X in 500µL PBS), followed by incubation at room temperature for 10 minutes in the dark. The cell suspension was transferred into a flow cytometer tube and its cell cycle profile was read using flow cytometry. The cell cycle profile was analyzed by ModFit LT 3.0.

Immunocytochemistry

Immunocytochemistry was developed (Cozmei, *et al.*, 2002) with some modifications. HeLa cells were grown with the density of 5×10^4 cells/cover slip in 24-well plate for 24 h prior to treatment of EFC, cisp, and combination of both. Cells were treated for 15 h and then fixed with cold methanol for 10 minutes and washed twice using PBS and sterile water. Cells were blocked with H_2O_2 and then pre-diluted blocking serum was added, continued with incubation for 10 minutes. Cells were washed using PBS and incubated with primary monoclonal antibody anti-p53 (*mouse monoclonal anti-p53 antibody*, BioGenex) overnight. Cells were washed using PBS, then universal detection kit was added (Star Trek Universal HRP Detection Kit, Ref STUHRP 700L10-KIT, Biocare Medical). Cells were then incubated with biotinylated universal secondary antibody for 20 minutes, followed by streptavidin-enzyme horse radish peroxidase for 10 minutes. Substrate solution chromogen 3,3'-diaminobenzidine (DAB) was exposed for 10 minutes and cells were counterstained with Mayer's Haematoxylin (Dako) for 1 minute. Then, cells were fixed with ethanol and xylol. Between each immunostaining step, cells were washed briefly in PBS pH 7.4. Negative controls were prepared by replacing the primary antibody with PBS. The p53 expression on the cells was observed by binocular microscope (Nikon YS 100, Japan).

Statistical analysis

Single and combinational cytotoxicity assay:

IC_{50} value was calculated by equation $y=Bx+A$ acquired from linear regression between concentration and % cell viability. IC_{50} is concentration inhibiting 50% cell proliferation. Cell viability was calculated with the formula (Meiyanto, *et al.*, 2011):

$$\% \text{ Cells viability} = \frac{\text{Treated cells abs} - \text{medium control abs}}{\text{Cells control abs} - \text{medium control abs}} \times 100\%$$

The datum were performed as average \pm SE from triplicate ($n = 3$) experiments.

Cell viability and p53 expression were statistically analyzed using one way ANOVA and statistical significance was estimated by using Tukey HSD. Statistical significance was determined at $p < 0.05$.

RESULT

Cytotoxic EFC and Its Combination with Cisp on HeLa cells

Cytotoxicity assay was used to evaluate the potential of EFC cytotoxicity on HeLa cells. Furthermore, IC_{50} value was acquired as the parameter of EFC concentration to inhibit 50% HeLa cells' growth. Fig. 1 shows the effect of EFC to cell viability for 24 h treatment. EFC caused inhibition on cell growth in a concentration-dependent manner (Fig. 1A). We suggest that EFC was found to be cytotoxic on HeLa cells with IC_{50} value of 65 ± 1.5 µg/mL. This fraction exhibited potent cytotoxicity ($IC_{50} \leq 100$ µg/ml) (Prayong, *et al.*, 2008). Treated cells showed cytotoxic effect with changes in cell morphology and the decrease of cell viability. Viable cells exhibited epithelial shape, but after being treated with certain concentration of samples, the cells exhibited cell shrinkage and rounding (Fig. 1B).

Cisp performed cytotoxic activity on HeLa cells with IC_{50} value of 16 µM (data not showed). IC_{50} value was then used to determine the concentration in combinational treatment that is concentration lower than IC_{50} value. Various

concentrations of cisp (2, 4, 8 μ M) were combined with 28 μ g/mL EFC. Fig. 2 sections confirm that combinational treatment performed morphological changes and caused decrease of the cells number. Cell viability measurement of the combination group (EFC combined to cisp) was considered significantly different compared to cisp-single treatment group ($p < 0.05$). At 8 μ M cisp in combination with 28 μ g/mL EFC decreased significantly HeLa cells' viability compared to 2 and 4 μ M cisp ($p < 0.05$).

There was approximately 63% decrease in HeLa cells' viability treated by 28 μ g/mL EFC combined 8 μ M cisp for 24 h. Cell viability acquired from single treatment of EFC and cisp above 100% was considered as 100%. Therefore, both low-concentration of EFC and cisp showed no inhibitory effect on HeLa cells. Whereas, combination between EFC and cisp caused a significant decrease in cell viability. This result indicates that EFC can be used in combination with cisp on HeLa cells.

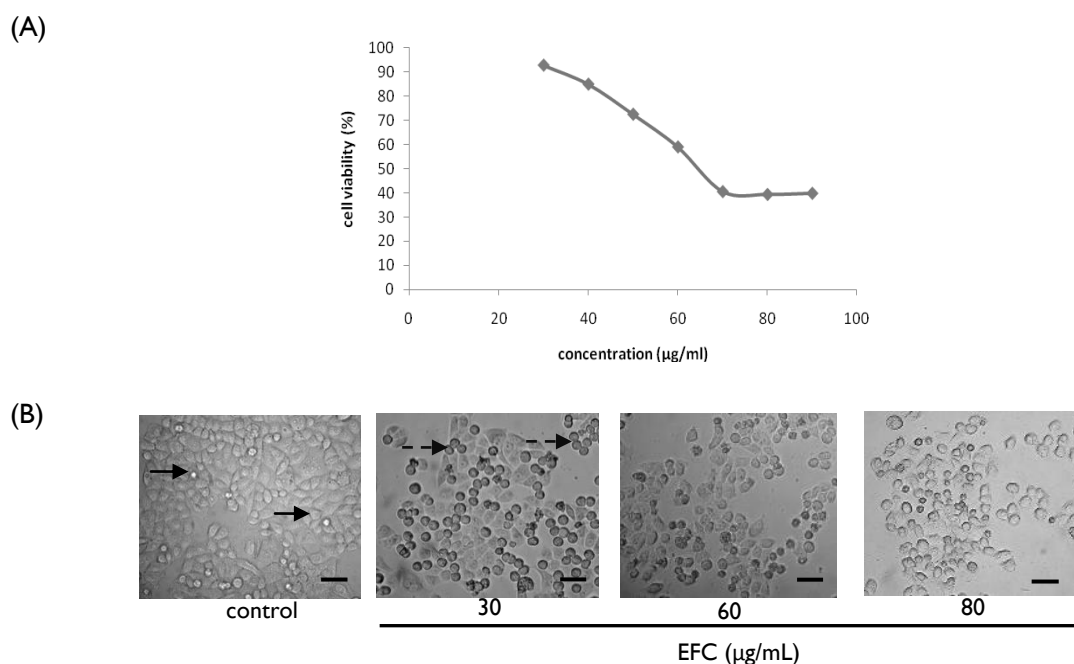


Figure 1. Effects of EFC on cell viability and morphological changes on HeLa cells. (A) HeLa cells was treated using EFC (0,30-90 μ g/mL) for 24 h and determined by MTT assay as described in materials and methods. Data are representative of values from three independent experiments. (B) Morphological changes of HeLa cells were observed using binocular microscope. Viable cells (full arrow), morphological changes (dotted line), bars: 50 μ M (magnification 100x)

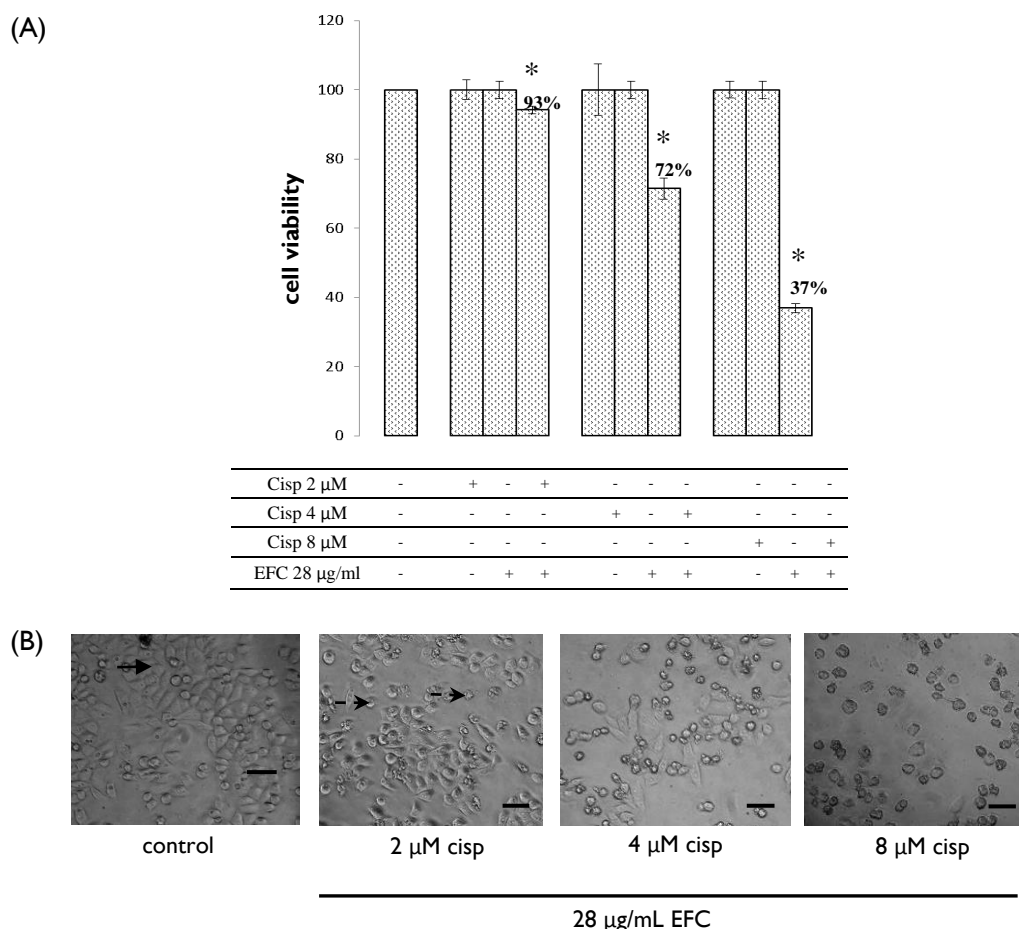


Figure 2. Cytotoxic Effect of EFC was combined with cisp on HeLa cells. (A) HeLa cells were treated for 24 h with combination both of EFC and cisp caused decreasing percentage of cell viability. Cells viability were determined by MTT assay as described in materials and methods. (*) Significances ($p < 0.05$). (B) Cells' morphological changes treated with combination both of EFC 28 μg/ml and cisp (2, 4, 8 μM) were examined and photographed by binocular microscope. Viable cells (full arrow), morphological changes (dotted line), bars: 50 μM (magnification 100x).

Effect of EFC and Cisp on Cells Death Induction on HeLa Cells

According to the cytotoxic combination between EFC and cisp mentioned below, cell death detection was carried out by flow cytometry using the less of IC₅₀ concentration. Fig. 3 shows the effect of EFC, cisp and combination of both caused cell death induction. EFC could not increase cell death through apoptosis signal. However, it could increase necrosis cell death which was induced by combination of EFC and cisp compared to cisp-single treatment. Cisp-single treatment and

combination of cisp with EFC (24 h treatment) caused 82% and 72% cell death, respectively (Fig. 3B). The apoptosis value of cisp-single treatment and its combination with EFC were 71.50% and 40.41%, while the necrosis values were 10.38% and 31.81%, respectively. EFC-single treatment caused less cell death induction with apoptosis value of 5.85% and necrosis value of 2.38%. These results suggest that EFC may affect HeLa cells' sensitivity to cisp which is caused by apoptosis and necrosis cell death.

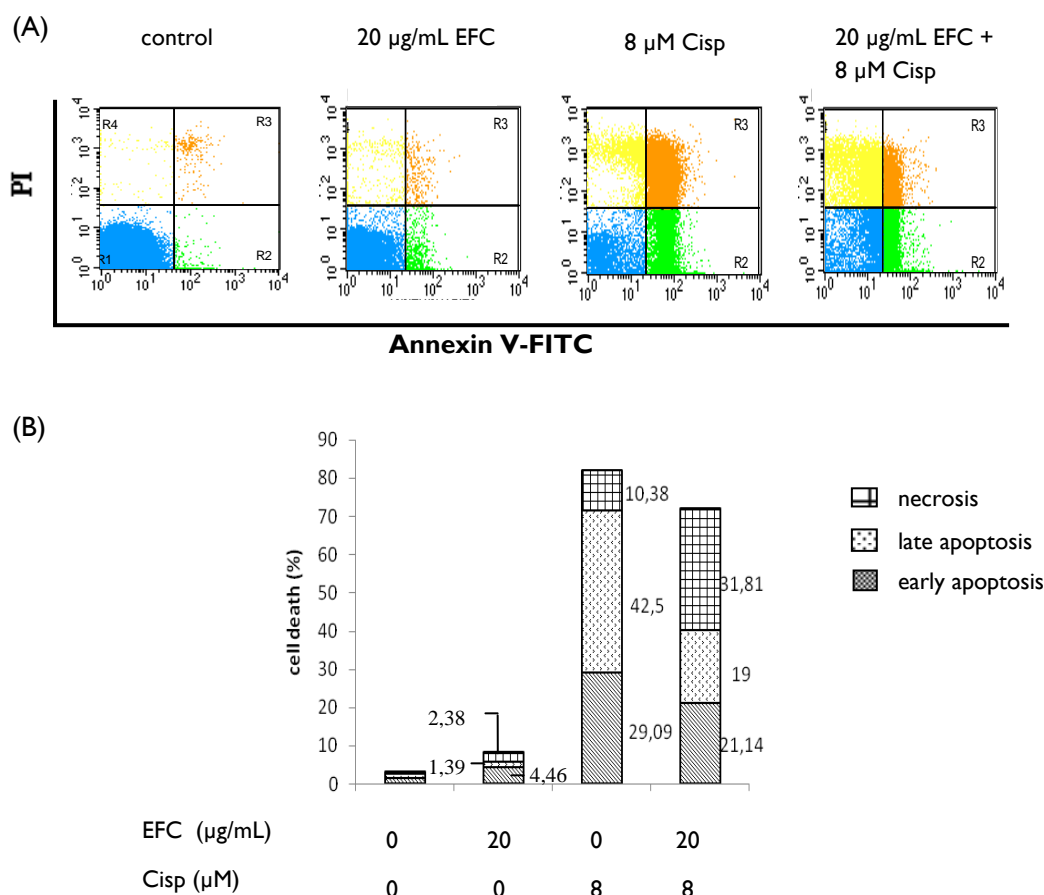


Figure 3. Cells death induction was caused by Cisp and its combination with EFC. (A) HeLa cells was treated with EFC 20 µg/mL, Cisp 8 µM, and both of it for 24 h. Then, Cells death was calculated by Flow cytometry assay using annexin V-FITC/PI staining. R1 viable, R2 early apoptotic, R3 late apoptotic, R4 necrotic cells. (B) Data was analyzed using Microsoft excel 2007 describing cell death percentage.

Effect of EFC and Cisp on Cell Cycle Modulation of HeLa Cells

Cell Cycle analysis by flow cytometry was used to evaluate the cell cycle distribution by EFC, cisp and combination of both. Cisp has been reported to induce cell cycle arrest in G2/M phase in some cancer cells (Fehrmann and Laimins, 2003) and this study confirm that EFC induced cell cycle arrest in S phase. Cell distribution analysis was performed to investigate the effect of EFC addition to cisp on cell cycle arrest of HeLa cells. Fig. 4 shows HeLa cells treated with 28 µg/mL EFC, 8 µM cisp, and a combination of the two agents for 24 h. HeLa cells

treated with EFC and cisp showed cells accumulation in S phase and G2/M phase, respectively. While combination of both agents caused redistribution of the cell cycle in G1 and S phase compared to cisp-treated cells. In addition, accumulation of cells in G2/M phase decreased compared to cisp-treated cells. These results above suggested that EFC enhances cytotoxicity induced by cisp on HeLa cells through cell cycle arrest in G1 and S phase. The cells progression in G1 and S phase might be caused by some proteins that play a role in cell cycle checkpoints.

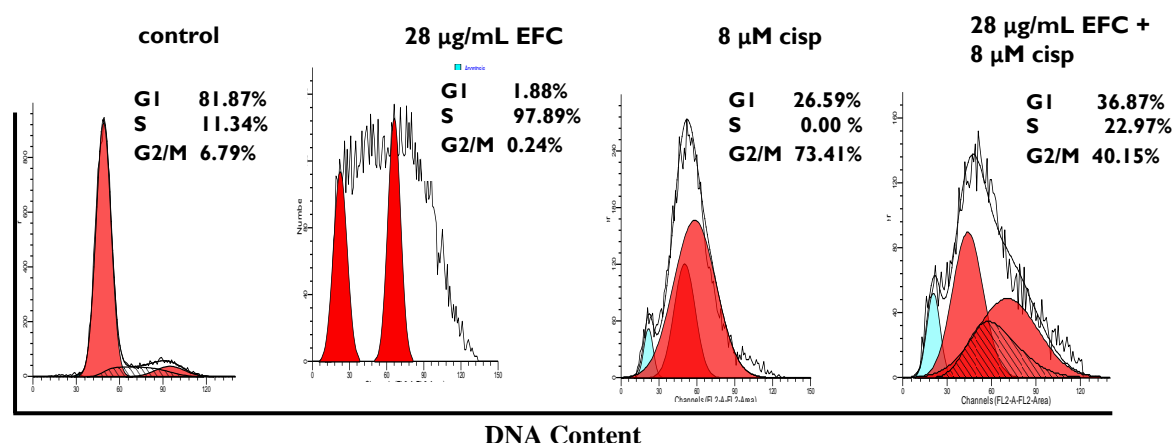


Figure 4. Cisp in combination with EFC caused cell cycle modulation on HeLa cells. Cells were treated with 28 µg/mL EFC, 8 µM cisp and combination of both for 24 h. Cell cycle distribution was calculated by Flow cytometry then analyzed using ModFit LT 3.0.

Expression of p53 was caused by EFC and Cisp on HeLa Cells

To confirm the mechanism of EFC and its combination with cisp in cell cycle arrest, therefore molecular target protein p53 was investigated. HeLa cells performed degradation and function loss of p53 as tumor suppressor protein which then cause uncontrolled proliferative cells (Fehrmann and Laimins, 2003; Minaguchi, *et al.*, 1998). As shown

in Fig. 5, immunocytochemistry evaluation indicated that p53 level increased significantly ($p < 0.05$) (Fig. 5A) confirmed by an intensive brown color in nucleus after being treated with 28 µg/mL EFC-single treatment and in combination with 8 µM cisp compared to cisp-single treatment and non-treated cells. The results suggest that EFC enhances cisp cytotoxicity on HeLa cells through p53 stabilization that perhaps play a role in cell cycle checkpoints.

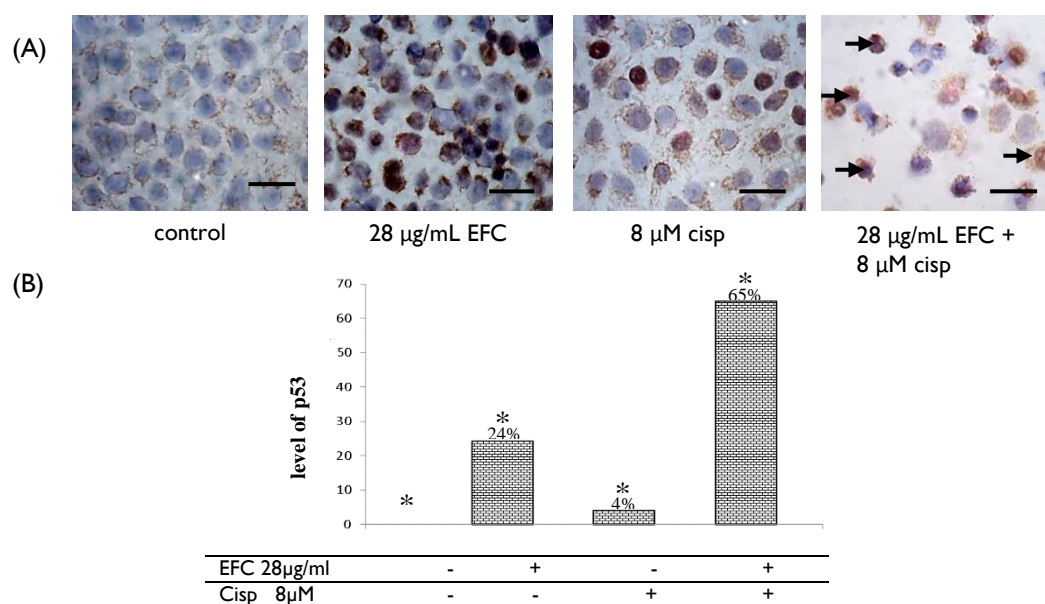


Figure 5. Effect of EFC and Cisp on p53 expression of HeLa cells. (A) HeLa cells were treated for 15 h with 28 µg/mL EFC, 8 µM cisp, and in combination of both. Data was examined and photographed by binocular microscope. Bars: 5 µM (magnification 400x). (B) Quantitative results showed analysis of p53 level in nucleus considered significantly different between combination treatment and single treatment ($p < 0.05$).

DISCUSSION

Ethyl acetate fraction of *C. sappan* (EFC) performed more potent cytotoxic effect on HeLa cells compared to ethanolic extract (320 µg/mL, data not shown), showing a concentration dependent manner, with IC₅₀ of 65±1.5 µg/mL. Combination treatment of EFC and cisp at low concentration performed significant inhibition of cell viability compared to cisp-single treatment. A relatively low concentration was used to minimize the additional side effects and cellular resistance of cisp. Decreasing cells viability might be due to either cell death or cell cycle arrest.

This study shows that combination of EFC and cisp treatment on HeLa cells induced more necrotic rate up to 31.81% compared to cisp-single treatment. Afterward, EFC-single treatment caused less apoptosis compared to cisp. Hence, cisp is possibly more responsible in inducing apoptosis than EFC and its combination with EFC. The combination between EFC and cisp may affect on cell cycle modulation that can cause a decrease in cell viability on HeLa cells. Cisp has been reported as anti-cancer agent by inducing G2/M phase arrest and apoptosis in some type of cancer cells (Jamieson and Lippard, 1999; Fehrman and Laimins, 2003). Our results suggest that EFC enhanced HeLa cells' sensitivity to cisp through cell cycle redistribution, causing arrest in G1 and S phase. Cell cycle arrest is related to the efficacy of cytotoxicity of chemotherapeutic agent being used. Previous studies reported chemo-sensitization of cancer cells by many chemotherapeutic drugs perhaps due to cell cycle modulation (Lawrence, *et al.*, 2003). Therefore, the delay of cell cycle distribution in G1 and S phase by EFC may be important in the sensitization of HeLa cells to cisp which causes a delay in G2/M phase. The experiments was continued to investigate the protein target on cell cycle by detecting the presence of p53.

HeLa cells are characterized with p53 degradation due to the binding of HPV E6 protein complexes to cellular factor E6AP which leads to ubiquitin-mediated degradation by proteasome (Buitrago-pérez, *et al.*, 2009). EFC increased p53 expression in nucleus and its combination with cisp significantly increased p53 expression compared to EFC and cisp-single treatment (Fig. 5). The p53

protein activation may indirectly affect cellular response through some protein regulation causing cell cycle arrest to lead DNA repair or cell death activation (Jamieson and Lippard, 1999; Ricci and Zong, 2006; Schmitt, *et al.*, 2002). These results suggested that the mechanism of EFC in sensitizing HeLa cells to cisp was possibly via p53-dependent. Brazilein as the main compound of *C. sappan* probably could inhibit HPV E6 interaction with E6-binding protein E6AP. Therefore, brazilein may be amendable as HPV inhibitor agent and as competitor of vaccine to prevent the development of cervical cancer. However, this hypothesis still needs further investigation by *in silico* study.

CONCLUSION

In conclusion, EFC exhibits potential ability to enhance sensitivity of cervical cancer HeLa cells to cisp *in vitro* through cell cycle arrest with p53 stabilization. Further molecular target detection to investigate its cellular pathway needs to be conducted.

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